Archives of Insect Biochemistry and Physiology 54:95–109 (2003)

Novel Diterpenoids and Hydrocarbons in the Dufour Gland of the Ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae)

Ralph W. Howard,[1]* James E. Baker,[1] and E. David Morgan[2]

Chemical constituents contained in the Dufour gland of the ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) were characterized. Three terpenes, β-springene, a homo-β-springene, and a homo-geranyllinalool constitute approximately 37% of the gland components, with the remaining 63% all being hydrocarbons. The hydrocarbons consist of a homologous series of *n*-alkanes (*n*-C_{21} to *n*-C_{31}), a trace amount of 3-methyl C_{23}, a homologous series of internally methyl-branched alkanes (11-methyl C_{23} to 13-methyl C_{35}), one dimethylalkane (13,17-dimethyl C_{33}), a homologous series of monoenes ($C_{25:1}$ to $C_{37:1}$) with the double bonds located at Δ^9, Δ^{13} and Δ^{15} for alkenes of carbon number 25 to 31 and at Δ^{13} and Δ^{15} for carbon numbers 33 to 37 and three homologous dienes in very low amounts with carbon numbers of 31, 32, and 33. The terpenoid and hydrocarbon composition of the Dufour gland was similar in virgin and mated females. However, in contrast to the hydrocarbons, the amount of β-springene and homo-geranyllinalool increased significantly with time after adult emergence from the cocoon. Although many hydrocarbons in the Dufour gland are the same as those on the cuticle of this species [Howard and Baker, Arch. Insect Biochem. Physiol. 53:1–18 (2003)], substantial differences also occur. Of particular note is the chain length of alkenes and location of the double bonds: cuticular alkenes have a chain length of C_{23} to C_{29} and double bond locations at Δ^5, Δ^7, and Δ^9, whereas the Dufour gland alkenes contains a greater range of carbon numbers and have no Δ^5 or Δ^7 alkenes. The Dufour gland contains only one of the long-chain dimethylalkanes found on the cuticle. Also, no terpenoids are found on the cuticle, and the Dufour gland contains none of the secondary wax esters that are major components on the cuticle. GC-MS analysis of lipids carried in the hemolymph of *H. hebetor* indicated that all hydrocarbons found on both the cuticle and in the Dufour gland are present, as are some of the wax esters. However, none of the terpenoids were detected in the hemolymph. This suggests that the hydrocarbons are synthesized in other tissues or cells, probably by oenocytes, and differentially partitioned between the cuticle and the Dufour gland. The terpenoids are most likely synthesized within the Dufour gland. Analysis of surface lipids from eggs laid within 18 h indicated that no diterpenoids were present. Rather, the lipids present on the eggs were *n*-alkanes, monomethylalkanes, alkenes, and secondary alcohol wax esters. This composition did not reflect that of the Dufour gland, hence eggs are not being coated with Dufour gland components during oviposition. Arch. Insect Biochem. Physiol. 54:95–109, 2003. Published 2003 Wiley-Liss, Inc.†

KEYWORDS: Dufour gland; venom apparatus; diterpenes; homo-diterpenes; homo-diterpene alcohols; hydrocarbons; semiochemistry; ectoparasitoids

[1]USDA-ARS-GMPRC, Manhattan, Kansas

[2]Keele University, Department of Chemistry, Keele, Staffordshire, United Kingdom

*Correspondence to: Ralph W. Howard, USDA-ARS-GMPRC, 1515 College Avenue, Manhattan, KS 66502. E-mail: howard@gmprc.ksu.edu

Received 26 March 2003; Accepted 26 June 2003

DOI: 10.1002/arch.10104
Published online in Wiley InterScience (www.interscience.wiley.com)

INTRODUCTION

Dufour Gland Secretions in Hymenoptera contain a broad array of chemicals that have been implicated in a number of physiological and semiochemical functions (Blum 1985; Haynes and Birch, 1985; Ali and Morgan, 1990). The Dufour gland secretions of many aculeates, including ants, bees, and wasps, are particularly well studied (Billen and Morgan, 1998), whereas much less information is available for parasitic Hymenoptera (Jervis and Kidd, 1996; Quicke, 1997). There is evidence that Dufour gland secretions in some braconids have oviposition deterrent effects (Vinson and Guillot, 1972; Guillot et al., 1974) and serve as gender-specific contact recognition pheromones (Syvertsen et al., 1995), and that components of the Dufour gland secretion can mediate oviposition responses in an ichneumonid (Mudd et al., 1982; Marris et al., 1996). We recently reported our findings on the morphology and chemistry of Dufour glands in *Cephalonomia tarsalis* (Ashmead) and *C. waterstoni* (Gahan) (Bethylidae) and in *Anisopteromalis calandrae* (Howard) and *Pteromalus cerealellae* (Ashmead) (Pteromalidae) (Howard and Baker, 2003b). These four cosmopolitan ectoparasitoids are important biological control agents that attack the major insect pests of stored grain in North America. In this article, we report on the Dufour gland chemistry of another major ectoparasitoid of stored product pests, the braconid *Habrobracon hebetor* (Say), which attacks pyralid moth pests in this ecosystem.

MATERIALS AND METHODS

Insects

Habrobracon hebetor from a strain collected in Dickinson county, Kansas, in 1998, was reared on wandering stage larvae of the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). Host larvae were maintained at 25°C and 55% RH on a diet of cracked wheat, wheat shorts, wheat germ, brewer's yeast, glycerin, and honey (McGaughey and Beeman, 1988). Newly emerged virgin adults of *H. hebetor* were obtained by isolating cocoons containing wasp pupae into 13 × 100 mm test tubes with plastic caps and observing daily for adult emergence (Howard and Baker, 2003a).

Dissection of Dufour Gland

Adult females were chilled on ice and dissected in a black depression plate under 0.9% NaCl containing 0.05% Triton X-100. Generally, the intact venom apparatus (Bender, 1943) can be obtained by grasping the thorax with one pair of forceps and by gently pulling the ovipositor with a second pair of forceps until the complex breaks free from the abdomen. The venom reservoir, surrounded by the venom gland filaments, is attached to the oviduct by a long duct. By removing the venom reservoir, hindgut, ovarian system, and the terminal ganglion of the ventral nerve cord, and by separating the valvulae that make up the ovipositor/stinger, the Dufour gland can be exposed. The tough, flattened, pouch-like gland is found near the base of the ovipositor between the two 2nd valvifers. In adults we examined, the gland is 0.2 to 0.25 mm in length. The Dufour gland has a short duct that inserts into the common oviduct. By pinching the duct with a fine pair of forceps, the gland can be removed intact. For GC-MS analysis, isolated glands were either analyzed individually by macerating the gland onto a Solid Phase Microextraction (SPME) fiber, or by extracting groups of glands that had been transferred to a small (approximately 1 × 3 mm) piece of filter paper (Howard and Baker, 2003b). Only quantitative differences were observed between the two extraction techniques.

Analysis of Hemolymph Lipids

Female wasps were chilled on ice, decapitated, gently squeezed on the thorax, and the resulting small drop of clear, yellowish hemolymph quickly absorbed onto the edge of a 1 × 3 mm piece of filter paper before coagulation occurred. Twenty wasps per replicate were so treated and two replicates were prepared with adults from separate cultures. For each replicate, the filter paper was placed

in a 50-µl insert in a GC Teflon crimp-cap vial, 4 µl hexane added, 2 µl removed with a GC syringe and subjected to GC-MS analysis using the same conditions as for the Dufour gland analyses. Although we took care not to contact the cuticle during collection of hemolymph, it is possible that the samples may contain a few fat body cells.

Analysis of Surface Lipids From Freshly Oviposited Eggs

Female wasps were held with wandering stage *P. interpunctella* larvae and allowed to sting and oviposit ad libitum overnight. The next day, eggs were carefully removed from the hosts and placed in a 1-ml beaker. Iso-octane (5 µl) containing 57 ng C_{20}/µl was added, the eggs were rinsed for 30 sec and then 3 µl was removed with a GC syringe and subjected to GC-MS analysis using the same conditions as for the Dufour gland analyses. Two replicate analyses were conducted, one with 60 eggs and one with 150 eggs.

Chemical Analyses

Electron impact mass spectral analyses were conducted with a Hewlett-Packard 5790A gas chromatograph (GC) (Hewlett-Packard, Inc., San Fernando, CA) containing a DB-5 bonded phase capillary column (15 m × 0.25 mm id, 0.2 µm film thickness) (J and W Scientific, Folsom, CA) connected to a Hewlett-Packard 5970 mass selective detector and a Hewlett-Packard 9133 data system. Ultrapure helium was the carrier gas, with a column head pressure of 0.75 kg/cm^2. Mass spectra were obtained at 70 eV. Analyses were temperature programmed with an initial temperature of 100°C, a final temperature of 320°C, a program rate of 5°C/min, and a 20-min final hold period. The splitless injector was set at 275°C and the GC/MS interface was at 280°C. Retention times of each diterpene and hydrocarbon component and equivalent chain length values (ECL) were obtained by comparison with known *n*-alkane standards (Howard et al., 1978). Hydrocarbon components were identified from their characteristic EI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978) in conjunction with equivalent chain length values.

Individual Dufour gland analyses were conducted with a 7 µm polydimethylsiloxane bonded phase fiber in a Supelco SPME holder (Supelco, Inc., Bellafonte, PA). The absorbed compounds were analyzed by GC-MS using the same parameters as listed above, with the exception that the fiber was desorbed for 2 min at 280°C with the septum purge closed before beginning the temperature program.

Double bond locations in alkenes were obtained by preparing vicinal thiomethyl ethers and examining their electron impact mass spectra (EI-MS) (Francis and Veland, 1981). Stereochemistry of the parent alkene was established from Fourier transform infrared analyses. Fourier transform infrared vapor-phase spectra on the underivatized alkenes were obtained on a Hewlett-Packard 5890 GC with a 5965B FT-IR detector and a 7958A data system. A DB-5 bonded phase capillary column (20 m × 0.25 mm id, 0.2 µm film thickness) using chromatographic conditions identical to those described above was used.

Effect of Age and Mating Status on Dufour Gland Composition

Dufour glands were dissected from female *H. hebetor* that were either 0 (newly emerged), 3, or 6 days old, and that were either mated or virgin. All females (except newly emerged females) had access to *P. interpunctella* larval hosts. Groups of 5 newly emerged virgin females were placed in 3 × 8 cm plastic snap-cap vials with screen lids and held for 3 or 6 days. Vials with mated females contained 2 male wasps. Two replicates of each treatment were prepared with two different cultures (separated by two generations) so that a total of four replicates of each age/mating status combination were analyzed. At appropriate time intervals, the vials were chilled in a refrigerator, then taken out and the females removed and dissected. Dufour glands from the five females in a treatment were pooled onto a 1 × 3 mm strip of filter paper, the

strips placed in 50-µl inserts in GC Teflon crimp-cap vials, and the vials held at –80°C until analysis. Iso-octane (5 µl) containing 57 ng/µl C_{20} as an internal standard was added to each vial just before analysis, and a volume equal to1 gland-equivalent was removed and analyzed by GC-MS as described above.

Voucher Specimens

Specimens of *H. hebetor* have been placed in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Manhattan, KS.

RESULTS

The volatile contents of the Dufour gland of *H. hebetor* were found to consist of a diterpene, β-springene, previously characterized from several mammals and a reptile (Burger et al., 1978, 1981; Avery et al., 1993; Waterhouse et al., 1996, 2001; Ibrahim et al., 1998). In addition, a homoditerpene that appears to be closely related to the β-springene, a tertiary alcohol that appears to be a homo-geranyllinalool, and 41 non-terpenoid hydrocarbons were also present (Fig. 1, Table 1). Peak A of Figure 1 is β-springene, and Figure 2A is the EI-MS and Figure 2B the vapor phase FT-IR of this diterpene. A molecular ion at m/z 272 ($C_{20}H_{32}$) is evident and the fragmentation pattern agrees closely with the published mass spectrum of β-springene (Burger et al., 1978), and with a mass spectrum of an authentic sample obtained from Prof. Peter Baekstrom Royal Technical Institute, Stockholm, and analyzed by one of us (E.D.M.) several years ago. The molecular formulae requires five unsaturation equivalents, and all of these must be olefinic rather than rings since no major ion fragments of high mass are present (Budzikiewicz et al., 1964). Diimide reduction of the homo-geranyllinalool produced a saturated product whose EI-MS spectrum showed a weak M-15 ion at m/z 297, a slightly larger M-29 at m/z 283 and a base peak of m/z 73. The ion at m/z 73 arises from α-cleavage to a protonated tertiary alcohol with methyl and ethyl substituents, consistent with the infrared data. In addition, this diterpene has an ECL of 19.20 on our DB-5 column, which corresponds exactly to the ECL value of the authentic β-springene that was analyzed earlier by one of us (E.D.M.) on a BP-1 capillary column (and hence would have very similar retention values when expressed as an ECL value). The vapor phase FT-IR

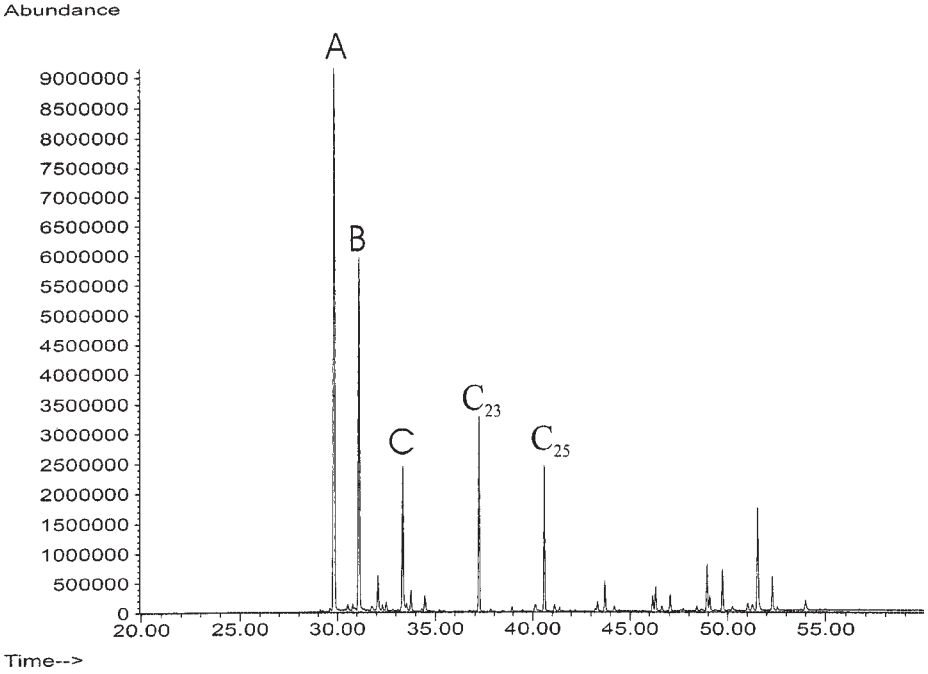

Fig. 1. Reconstructed TIC of the contents of the Dufour gland of *H. hebetor* desorbed from an SPME fiber. **A**: β-springene; **B**: homo-β-springene; **C**: homo-β-geranyllinalool. Small peaks between 29 and 34 min are likely artefacts resulting from thermal decomposition reactions of the diterpenes in the hot injection port of the GC. All other peaks are hydrocarbons.

TABLE 1. Identification and Composition of Components in the Dufour Gland of *Habrobracon hebetor*

Compound	ECL[a]	CN[b]	Diagnostic EI-MS ions[c]	Mean ng/gland (SE)[d]
β-springene	ca. 19.20	20	69, 272 (M^+)	61.1 (7.4)*
homo-β-springene	ca. 19.85	21	69, 286 (M^+)	33.1 (4.8)
homo-β-geranyllinalool	ca. 20.91	22	69, 304 (M^+)	30.3 (4.9)
n-C_{21}[e]	21.00	21	296	0.6 (0.1)
n-C_{22}[e]	22.00	22	310	0.3 (0.1)
n-C_{23}[e]	23.00	23	324	38.8 (3.9)
11-MeC_{23}[e]	23.33	24	169, 197, 323	0.4 (0.1)
3-MeC_{23}	23.70	24	309, 323	Tr[f]
n-C_{24}[e]	24.00	24	338	1.5 (0.2)
Z-9-$C_{25:1}$[e]; Z-13-$C_{25:1}$	24.80; 24.72	25	350 [173, 271; 215, 229; 444 (M^+)]	0.9 (0.2)
n-C_{25}[e]	25.00	25	352	41.8 (3.6)
13-MeC_{25}[e]	25.33	26	197, 351	1.2 (0.2)
n-C_{26}[e]	26.00	26	366	0.8 (0.1)
Z-15-$C_{27:1}$	26.72	27	378 [215, 257, 472 (M^+)]	0.4 (0.1)
Z-13-$C_{27:1}$	26.79	27	378 [229, 243, 472 (M^+)]	1.5 (0.2)
Z-9-$C_{27:1}$[e]		27	378 [173, 229, 472 (M^+)]	0.2 (0.1)
n-C_{27}[e]	27.00	27	380	12.9 (1.4)
13-MeC_{27}[e]	27.33	28	197, 225, 379	0.7 (0.1)
11-MeC_{28}	28.33	29	169, 267, 293	0.3 (0.1)
Z-15-$C_{29:1}$	28.70	29	406 [243, 257, 500 (M^+)]	3.6 (0.5)
Z-13-$C_{29:1}$	28.72	29	406 [229, 271, 500 (M^+)]	4.4 (0.6)
Z-9-$C_{29:1}$[e]	28.80	29	406 [173, 327, 500 (M^+)]	Tr
n-C_{29}[e]	29.00	29	408	2.8 (0.3)
13-MeC_{29}[e]	29.33	30	197, 253, 407	3.5 (0.5)
14-MeC_{30}[e]	30.33	31	211, 253, 421	0.4 (0.1)
$C_{31:2}$	30.60	31	432	1.2 (0.2)
Z-15-$C_{31:1}$	30.70	31	434 [257, 271, 528 (M^+)]	12.5 (1.6)
Z-13-$C_{31:1}$	30.72	31	434 [229, 299, 528 (M^+)]	3.2 (0.5)
Z-9-$C_{31:1}$	30.80	31	434 [173, 355, 528 (M^+)]	Tr
n-C_{31}	31.00	31	436	0.9 (0.1)
13-MeC_{31}[e]	31.33	32	197, 291, 435	12.6 (1.6)
$C_{32:1}$	31.72	32	448	Tr
12-MeC_{32}[e]	32.33	33	183, 309, 449	2.5 (0.3)
$C_{33:2}$	32.60	33	460	1.7 (0.3)
Z-15-; Z-13-$C_{33:1}$	32.72	33	462 [257, 299; 229, 327; 556 (M^+)]	33.8 (3.5)
13-MeC_{33}[e]	33.33	34	197, 309, 463	13.7 (1.4)
13,17-DiMeC_{33}[e]	33.55	35	197, 323, 253, 267, 477	1.5 (0.2)
$C_{35:2}$	34.60	35	488	0.4 (0.1)
Z-15-; Z-13-$C_{35:1}$	34.72	35	490 [257, 327; 229, 355; 584 (M^+)]	6.6 (0.9)
Z-15-; Z-13-$C_{37:1}$	36.72	37	518 [257, 355; 229, 383; 612 (M^+)]	Tr
Total gland contents	—	—	—	332.2 (37.1)

[a]Equivalent chain length.

[b]Carbon number.

[c]Diagnostic ions in brackets are for the dimethyl disulfide derivatives of the alkene.

[d]Based on 21 samples. No means were affected by mating status and only three means were affected by age.

[e]Compounds that are also present as part of the citicular hydrocarbons of this species (Howard and Baker, 2003a).

[f]Trace = less than 0.1 ng.

*Quantity of this compound varied with age: 26.2 ng (12.8) at 0 days (N = 5), 72.4 ng (10.1) at 3 days (N = 8) and 71.5 ng (10.1) at 6 days (N = 8). The mean at 0 days is significantly different ($P = 0.02$) from either 3 or 6 days, which are not significantly different from each other by LSD.

**Quantity of this compound varied with age: 7.4 ng (8.5) at 0 days (N = 5), 38.5 ng (6.7) at 3 days (N = 8) and 36.3 ng (6.7) at 6 days (N = 8). The mean at 0 days is significantly different ($P = 0.02$) from either 3 or 6 days, which are not significantly different from each other by LSD.

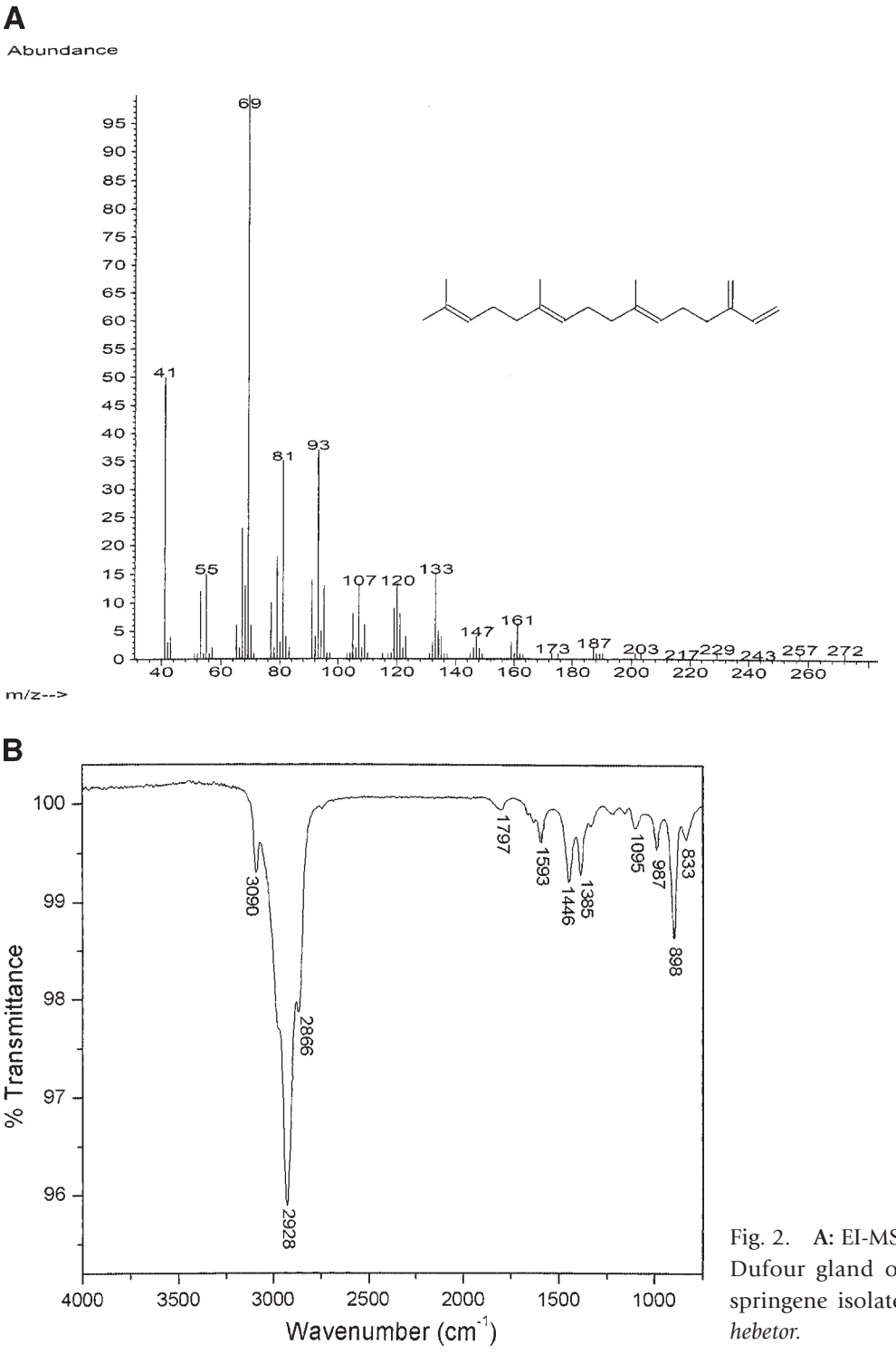

Fig. 2. **A:** EI-MS of β-springene isolated from Dufour gland of *H. hebetor.* **B:** FT-IR of β-springene isolated from Dufour gland of *H. hebetor.*

spectrum (Fig. 2B) is also consistent with the structure proposed, showing a strong absorption at 3,090 cm^{-1} for the pair of terminal vinyl C-H stretches (Nakanishi, 1962, Pouchert, 1989) and no bands consistent with any functionality other than olefinic units and alkyl units. The isomeric α-springene previously described from a springbok and the Dufour gland of the ant *Nothomyrmecia macrops* (Burger et al., 1981; Billen et al., 1988) has an ECL of 19.45 and a fragmentation pattern

that does not match our compound as well as the β-springene does. An authentic sample of the α-springene was also previously examined by GC-MS by one of us (E.D.M.). We do not have IR data for the α-springene but would anticipate that its absorption at approximately 3,090 cm^{-1} would be of lower intensity because it has only one set of terminal vinyl protons rather than two.

Peak B of Figure 1 appears to be a homo-β-springene with an ECL of 19.85 (Table 1). The mass spectrum of this compound (Fig. 3A) is very similar to that of β-springene but has a molecular ion

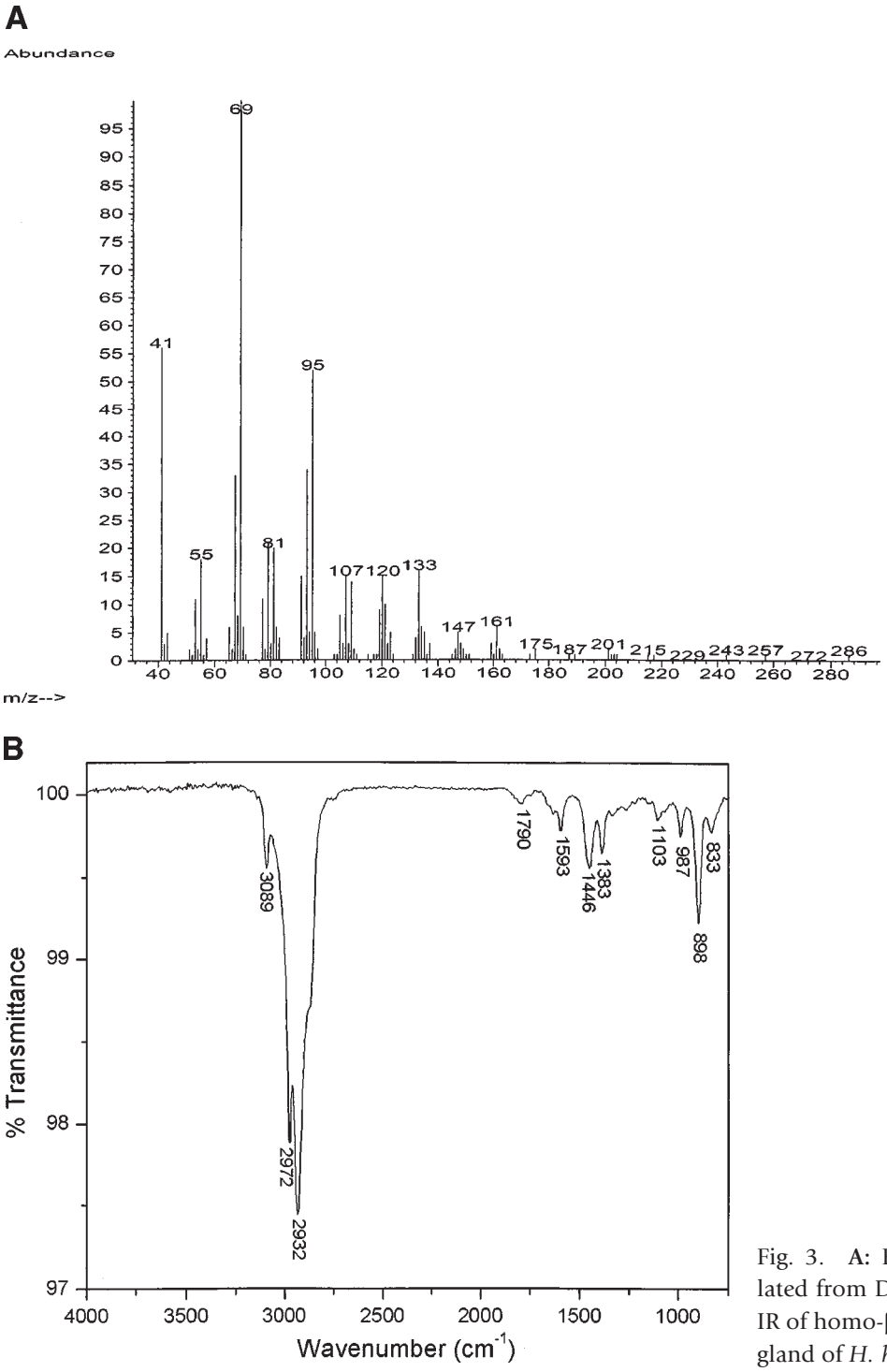

Fig. 3. **A:** EI-MS of homo-β-springene isolated from Dufour gland of *H. hebetor.* **B:** FT-IR of homo-β-springene isolated from Dufour gland of *H. hebetor.*

at m/z 286 ($C_{21}H_{34}$) indicating that it contains one additional methylene group, no additional unsaturation, and no ring structures. Because its vapor phase FT-IR spectrum (Fig. 3B) is nearly identical to that of the β-springene, the basic carbon skeleton of the two compounds is likely the same, including the presence of the two terminal vinyl groups. Assignment of the location of the added methylene group is not possible with our current data.

Peak C of Figure 1 appears to be a homogeranyllinalool. A weak molecular ion is present at m/z 304 (Fig. 4A) ($C_{21}H_{36}O$) and a somewhat stronger M-18 ion (loss of water) is present at m/z 286. This spectrum is very similar to that of geranyllinalool. The general appearance of the mass

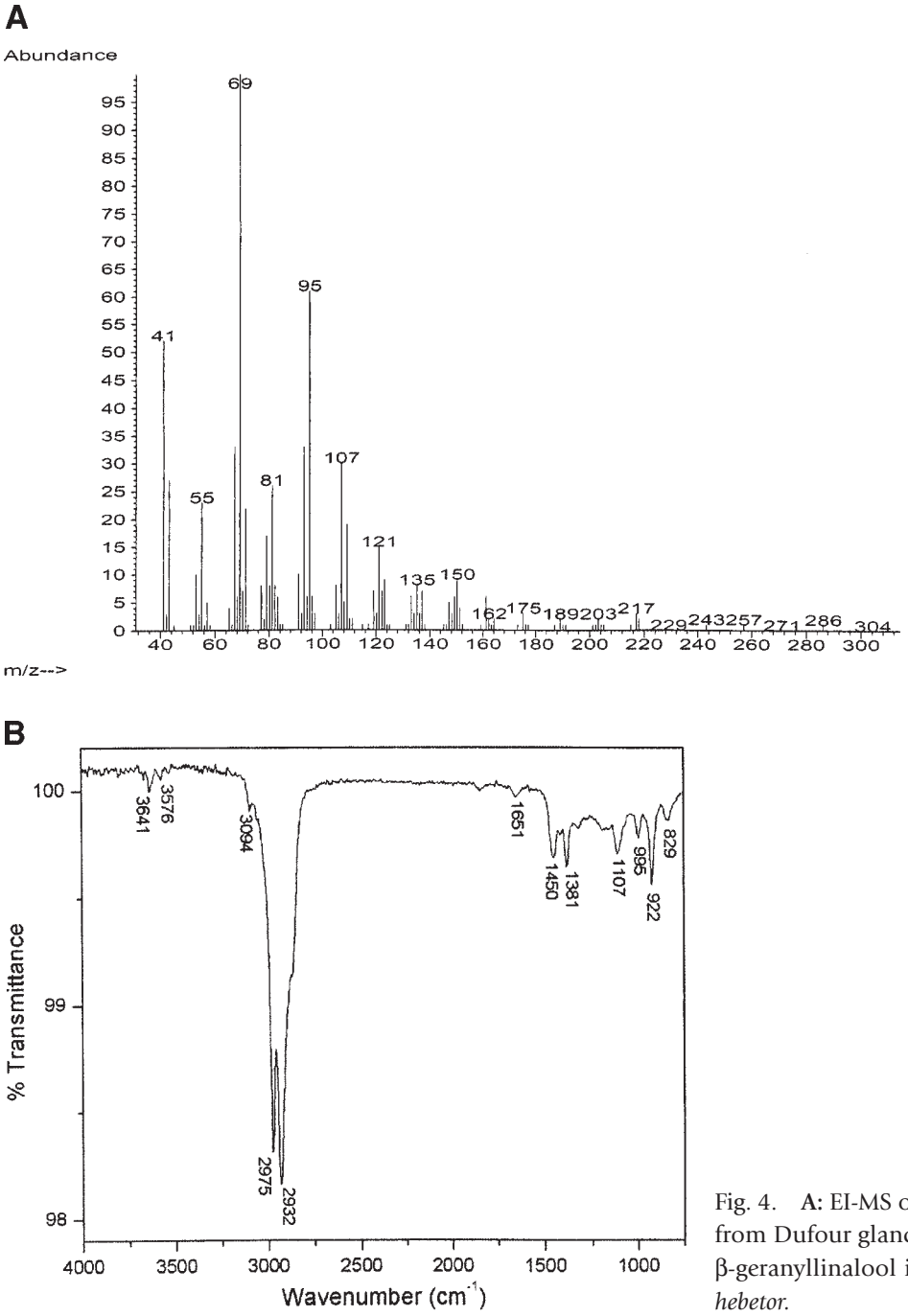

Fig. 4. **A:** EI-MS of homo-β-geranyllinalool isolated from Dufour gland of *H. hebetor.* **B:** FT-IR of homo-β-geranyllinalool isolated from Dufour gland of *H. hebetor.*

spectrum with a steady decrease in intensity of the ion fragments from low to high mass closely matches a linear diterpene structure rather than a cyclic one where higher mass ion fragments are prominent (Budzikiewicz et al., 1964). The hydroxyl function appears to be tertiary rather than primary as indicated by the split hydroxyl absorptions in the vapor phase FT-IR spectrum (Fig. 4B) at 3,641 and 3,576 cm^{-1} (Nakanishi, 1962; Pouchert, 1989). The model compounds linalool and nerolidol with a corresponding tertiary alcohol alpha to a olefin have vapor phase FT-IR split hydroxyl absorptions of 3,638 and 3,590 cm^{-1} and 3,636 and 3,590 cm^{-1}, respectively (Pouchert, 1989). Primary alcohols in model compounds with unsaturation on the alpha carbon (such as nerol, geraniol, and farnesol) show vapor phase FT-IR hydroxyl absorptions at 3,658 cm^{-1} (Pouchert, 1989). The terminal vinyl absorption at 3,094 cm^{-1} in peak C is about half the intensity of this band in β-springene and homo-β-springene, suggesting that the hydroxyl function has added to the interior member of the two terminal vinyls to give the tertiary alcohol rather than at one of the other double bonds, all of which have methylene groups alpha to the double bond. Diimide reduction of β-springene produced a saturated compound with a molecular ion at 282 ($C_{20}H_{42}$) and diagnostic electron mass spectrometry (EI-MS) fragment ions at m/z 113, 127, 183, 197, 253 and 267, consistent only with the carbon skeleton of β-springene. The model compound 4-methyl-1,6-heptadien-4-ol with a tertiary hydroxyl flanked by methylene groups has a vapor phase FT-IR hydroxyl absorption of 3,610 cm^{-1} (Pouchert, 1989). As with the homo-diterpene of peak B, we do not have sufficient data to assign the location of the additional methylene in this molecule.

The 41 non-terpenoid hydrocarbons consist of a homologous series of *n*-alkanes (C_{21} to C_{31}), a trace amount of 3-methyl C_{23}, a homologous series of internally methylbranched alkanes (11-methyl C_{23} to 13-methyl C_{35}), one dimethylalkane (13,17-dimethyl C_{33}), a homologous series of monoenes ($C_{25:1}$ to $C_{37:1}$) with the double bonds located at Δ^9, Δ^{13}, and Δ^{15} for alkenes of carbon number 25 to 31 and at Δ^{13} and Δ^{15} for carbon numbers 33 to 37, and three homologous dienes in very low amounts with carbon numbers of 31, 32, and 33 (Table 1). The locations of the double bonds in the dienes were not determined because of low component abundance. FT-IR analysis indicated that all alkenes and alkadienes were of Z-configuration [absence of a strong band at 970 cm^{-1} and presence of a weak band at 3,012 cm^{-1} and a weak to moderate band at 1,650 cm^{-1} (Nakanishi, 1962; Krokos et al., 2001)]. Based on the internal standard, the mean total quantity of compounds in each Dufour gland was 332 ng, with the terpenoids representing 125 ng per gland (approximately 37%) and the non-terpenoid hydrocarbons representing 208 ng (approximately 63%).

We considered it possible that the Dufour gland components of *H. hebetor* might serve a pheromonal function, and that the amount or composition of the secretion might be regulated by either mating status or age. Thus, we conducted a factorial analysis of the Dufour gland contents with mating status (virgin, mated) and age (0, 3, 6 days) being the factors of interest. Neither the terpenoids or the non-terpenoid hydrocarbons showed any variation as a function of mating status. The hydrocarbons also showed no significant changes in amount or percentage composition with age. However, β-springene and the homo-geranyllinalool did show significant variation with age: the 3- or 6-day-old females (approximately 72 ng β-springene and approximately 37 ng geranyllinalool) had significantly more of these two terpenoids ($P = 0.02$) than did 0-day-old females (approximately 26 ng β-springene and approximately 7 ng of the homogeranyllinalool).

All hydrocarbons found in the Dufour gland were present in hemolymph, but none of the three terpenoids were detected there. In addition, all of the hydrocarbons and some of the wax esters found on the cuticle of this wasp were found in the hemolymph (Fig. 5) (Howard and Baker, 2003a). However, the hemolymph hydrocarbons and wax esters were not present in the same proportions as those found on either the cuticle or in the Dufour gland.

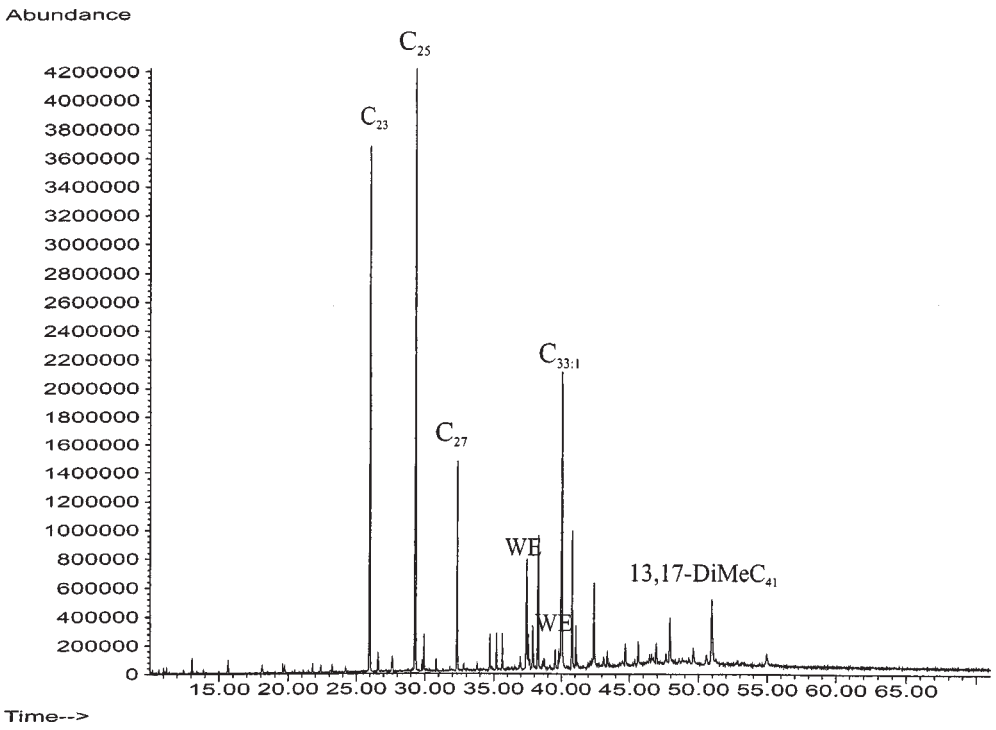

Fig. 5. Reconstructed TIC of lipids extracted from the hemolymph of *H. hebetor*. WE = wax esters.

The surface lipids extracted from freshly oviposited *H. hebetor* eggs did not mirror the contents of the Dufour gland (Fig. 6). No diterpenoids were found. Rather, the lipids consisted of *n*-alkanes (C_{25} to C_{33}), 12-, 13-, and 15-methylalkanes (Carbon number 29 to 37), a series of monoenes with the double bond located at either Δ^{13} or Δ^{15} (based on ECL values) and with carbon numbers of 31 to 39, and secondary wax esters similar to those found on the cuticle of adults (Howard and Baker, 2003a). These compositions more closely mirror that of hemolymph lipids than they do adult cuticular lipids.

DISCUSSION

Unlike the aculeate Hymenoptera where a rich literature exists on the chemistry and function of Dufour gland constituents (Ali and Morgan, 1990; Billen and Morgan, 1998), the literature on parasitoid Dufour gland chemistry is sparse. Only six species seem to have been characterized: *Venturia canescens* (Gravenhorst) (Ichneumonidae) (Mudd et al., 1982; Marris et al., 1996), *Cephalonomia tarsalis* and *C. waterstoni* (Bethylidae) (Howard and Baker, 2003b), *Anisopteromalus calandrae* and *Pteromalus cereallae* (Pteromalidae) (Howard and Baker, 2003b) and *Cardiochiles nigriceps* (Viereck) (Braconidae) (Syvertsen et al., 1995). The chemicals reported from the ichneumonid Dufour gland are a simple mixture of saturated and monounsaturated hydrocarbons: Z-8-, Z-9-, and Z-10-heneicosene, heneicosane, Z-10-tricosene, tricosane, Z-10-pentacosene and pentacosane with the Z-10-tricosene being the dominant component (62% of the total mixture). The Dufour gland chemistries of the two bethylids and two pteromalids also are dominated by hydrocarbons. The two bethylids contain *n*-alkanes, isomeric monoenes, 2-, 3-, and 5-methylalkanes and 5,X-dimethylalkanes (where X is a methyl branch located towards the interior of the alkyl chain). In addition, one of the bethylids, *C. waterstoni*, has 2,X-dimethyl alkanes, where X is 15 or 17 methylene units beyond the 2-methyl branch point. In contrast to other parasitoid taxa examined, the two pteromalids have no unsaturated hydrocarbons. Rather, they have complex mixtures of *n*-alkanes, monomethylalkanes with the methyl branch both close to the terminus of the carbon chain and located interiorly, a

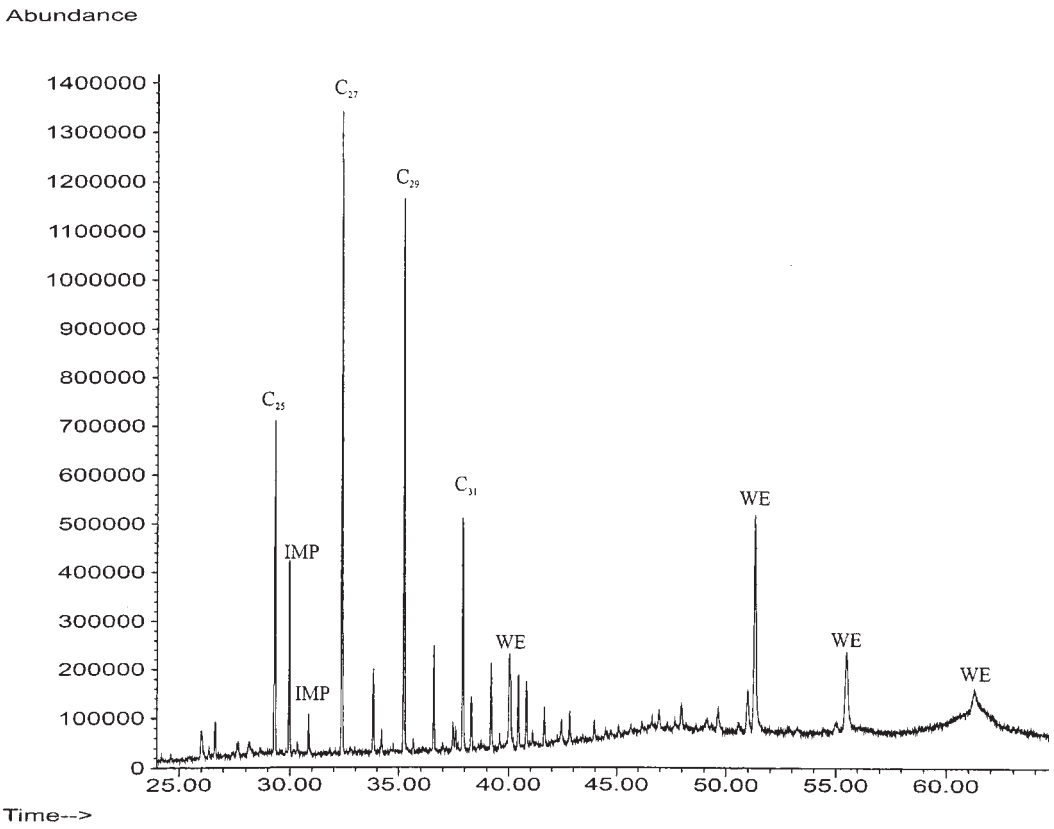

Fig. 6. Reconstructed TIC of lipids extracted from the surface of eggs of *H. hebetor*. WE = wax esters; IMP = phthalate impurity.

variety of classes of dimethylalkanes, trimethylalkanes and even tetramethylalkanes. *Pteromalus cerealellae* has, in addition, two long chain aldehydes, tetracosanal and hexacosanal. The Dufour gland chemistry of the braconid, *Cardiochiles nigriceps,* is composed solely of hydrocarbons, with a carbon number range of 23 to 35, consisting of a homologous series of *n*-alkanes, Z-monoenes and Z,Z-alkadienes, which are also reported to occur on the cuticle of this species. The dominant components of *C. nigriceps* appear to be *n*-pentacosane, Z-13- and Z-14-nonacosene, and Z,Z-7,15-nonacosadiene. The ichneumonid Dufour gland secretions function as conspecific egg marking pheromones preventing superparasitism (Marris et al., 1996), whereas the semiochemical function of the *C. nigriceps* Dufour gland secretion has been shown to be courtship mediation (Syvertsen et al., 1995). The function of Dufour gland chemicals in the other four species of parasitoids is unknown.

The chemistry of the Dufour gland secretions of *H. hebetor* is similar in some respects to the other parasitoids examined, but distinctly dissimilar in many other aspects. Quantitatively, hydrocarbons make up the major proportion of the secretion (63%), but unlike the other parasitoids, the composition of the Dufour gland hydrocarbons differs substantially from that of the cuticular hydrocarbons. Although alkenes are dominant components of both tissues, the carbon number distribution and double bond isomeric composition differ strikingly. The cuticle has shorter chain-length alkenes ($C_{23:1}$ to $C_{29:1}$) than does the Dufour gland ($C_{25:1}$ to $C_{37:1}$) and its isomeric composition consists exclusively of Δ^5-, Δ^7-, and Δ^9-alkenes. The Dufour gland has two sets of isomeric alkenes: for carbon numbers 25 to 31 the isomers are Δ^9-, Δ^{13}-, and Δ^{15}, and for carbon numbers of 33 to 37 only Δ^{13}- and Δ^{15} isomers occur. We know of no other case of such a striking difference in long-chain alkene

composition between different tissue sources in an insect. The Dufour gland has low quantities of dienes, whereas the cuticle has none. In addition to these differences, the cuticle has an extensive series of homologous long-chain dimethylalkanes, whereas the Dufour gland has only a single long-chain dimethylalkane (13,17-dimethyl C_{33}). Furthermore, the cuticle of *H. hebetor* has an abundant and extensive array of secondary alcohol wax esters (Howard and Baker, 2003a), whereas the Dufour gland has no wax esters.

The remaining portion of the Dufour gland chemistry of *H. hebetor* (approximately 37%) is terpenoid in nature. The major compound is β-springene, a diterpene that has been previously isolated from a diversity of organisms, including the American alligator (Ibrahim et al., 1998), the smooth-fronted caiman (Avery et al., 1993), a collared peccary (Waterhouse et al., 1996), the white-lipped peccary (Waterhouse et al., 2001), and two springboks (Burger et al., 1978, 1981). In insects, it has been found in the Dufour glands of the primitive Australian ant *Nothomyrmecia macrops* (Billens et al., 1988) and of the army ant *Aenictus rotundatus* (Oldham et al., 1994b) (where it is accompanied by α-springene) and as a trace component in the stingless bee *Nannotrigona testaceicornis* (Cruz-Lopez et al., 2001). Closely related compounds such as geranyllinalool have been identified in the Dufour glands of the soldier caste of the army ant *Eciton burchelli* (Keegans et al., 1993), and geranylgeraniol also occurs in *N. testaceicornis* (Cruz Lopez et al., 2001). In *H. hebetor*, β-springene is the most abundant individual compound in the Dufour gland. The remaining two terpenoids appear to be previously unreported and their complete structures remain tentative until further chemical analyses can be conducted. The lesser in quantity of these two additional components appears to be a homo-β-springene, and this is the first such report of a homo-diterpene in insects. The remaining component appears to be a hydroxylated derivative of the homo-β-springene (also previously unknown in insects), with the hydroxyl group being tertiary and located adjacent to the terminal vinyl group. The location of the additional carbon (probably a methyl group) on the two homo-compounds cannot be determined from the mass spectra or the infrared spectra, and initial experiments designed to selectively oxidize or reduce the structures to definable units have not been successful.

To a large extent, the hydrocarbons found in the Dufour glands of most of the parasitoids examined are also found on their cuticle, although several specific hydrocarbons in the Dufour glands of *C. waterstoni*, *A. calandrae*, *P. cerealellae*, and *H. hebetor* are not found as part of the cuticular complex (Howard and Baker, 2003b). The relative abundance or ratios of the hydrocarbons found in both Dufour gland and cuticular extracts for most of these species are similar (with the conspicuous exception of *H. hebetor*), suggesting that these chemicals perhaps arise from a common source. A similar correspondence in Dufour gland chemistry and cuticular chemistry has also been reported for several species of bumblebees (Oldham et al., 1994a), in polistine wasps (Dani et al., 1996), and in the honeybee *Apis mellifera* (Gozansky et al., 1997). Ants, however, while being shown to have a large repertoire of hydrocarbons in their Dufour gland secretions (Billen and Morgan, 1998), contain mostly short to medium length hydrocarbons that are not cuticular components.

To date, most evidence suggests that the long-chain hydrocarbons on both the cuticle and in specialized glands such as the Dufour gland arise from oenocytes in tissues associated with the haemocoel and integument. Oenocytes apparently secrete the hydrocarbons into the hemolymph where they are transported by lipophorin, a multifunctional plasma lipoprotein, and released either across cuticular membranes or released across membranes of specialized glands (Blomquist et al., 1998; Van der Horst et al., 1993; Schal et al., 1998a,b; Jurenka and Subchev, 2000). Gozansky et al. (1997) directly showed that in vitro incubation of the Dufour gland of the honeybee with 1-C^{14}-acetate did not result in biosynthesis of hydrocarbons in this gland, whereas injection of the labeled acetate into the whole organism and then isolating Dufour gland hydrocarbons did result in uptake of the la-

bel. Schal et al. (1998a) found a similar situation for the medium chain length hydrocarbons in the pheromone gland of females of the moth *Homomelina lamae* (Lepidoptera: Arctiidae). There, too, the gland itself was shown not to biosynthesize the pheromonal hydrocarbons but to merely serve as a reservoir, obtaining the hydrocarbons from the lipophorin shuttle.

Although we have not conducted experiments per se on tissue synthesis of either the cuticular or Dufour gland hydrocarbons and diterpenes of *H. hebetor*, we have conducted an indirect experiment to address the question of where these hydrocarbons are made. Our hypothesis was that if all hydrocarbons are made by oenocytes and carried by lipophorin to various target tissues as postulated by Schal et al. (1998a,b), then analysis of hemolymph lipids should reveal that both cuticle-specific and Dufour gland-specific hydrocarbons would be present. Such, indeed, was the case. Not only were all of the hydrocarbons present (albeit not in the same proportions as found either on the cuticle or in the Dufour gland) but also the secondary alcohol cuticular wax esters were found in the hemolymph extracts. No evidence was found, however, for the presence of terpenoids in hemolymph, suggesting that they are perhaps synthesized within the Dufour gland itself.

The semiochemical function, if any, of the constituents of the Dufour glands of *H. hebetor* remains to be determined. It seems a fair assumption that given the biochemical investment that the females have put into the production of the relatively substantial quantities of these chemicals, that they must serve some useful function in the behavioral/ecological relationships of the species. The noncuticular components that we detected in the Dufour glands of bethylids and pteromalids, particularly the long-chain aldehydes in *P. cerealellae*, are certainly prime candidates for a semiochemical function in these parasitoids (Howard and Baker, 2003b). As noted earlier, the Dufour gland components of the parasitoid *C. nigriceps* were postulated to serve as sex pheromones (Syvertsen et al., 1995), whereas those of *V. canescens* were postulated to serve a role in preventing superparasitism by closely related females (Marris et al., 1996). Both of these species are endoparasitoids, whereas all of the other species examined are ectoparasitoids. Hoffmeister (2000) postulated that the parasitoid *Halticoptera rosea* (Hymenoptera: Pteromalidae) applied a marking pheromone, derived either from the wasp's Dufour gland or poison gland to the surface of rose hips in which the host, a fly *Rhagoletis basiola*, had deposited its eggs in the fruit pulp. This marking pheromone was shown to deter further oviposition by conspecifics. Preliminary efforts in our laboratory to demonstrate similar behavioral roles for the *H. hebetor* Dufour gland secretions have been equivocal.

Bender (1943) postulated that the function of the Dufour gland secretion in *Habrobracon juglandis* (Hymenoptera: Braconidae) was to serve as a lubricant in aiding the passage of the egg along the ovipositor. A similar function has also been postulated by Robertson (1968) and Copland and King (1971), although the postulate was not experimentally confirmed in any of the cases. Our analysis of the surface lipids of the eggs of *H. hebetor* clearly shows that the Dufour gland components are not being used as a lubricant. None of the diterpenoids in the Dufour glands are found on the eggs. Furthermore, secondary wax esters are found on the egg surface, and these esters do not occur in the Dufour gland but rather only on the exocuticle of the female (Howard and Baker, 2003a). No chemicals derived from the larval host on which the eggs were removed were found. Considerable evidence now exists that cuticular hydrocarbons function in some cases as species and gender recognition cues (Howard, 1993) and it is possible that the Dufour gland hydrocarbons may be serving a similar role. The function of the diterpenoids remains unknown.

ACKNOWLEDGMENTS

We thank Dr. Larry Seitz, USDA-ARS, Manhattan, KS, for conducting the FT-IR analyses. We also thank Dr. Gary Blomquist, University of Nevada-Reno and Dr. David Stanley, University of Nebraska-Lincoln, for reviewing an earlier version of

this article. The mention of a proprietary product does not constitute an endorsement or recommendation for its use by USDA.

LITERATURE CITED

Ali FA, Morgan ED. 1990. Chemical communication in insect communities: a guide to insect pheromones with special emphasis on social insects. Biol Rev 65:227–247.

Avery JW, Shatagati A, Turner A, Wheeler JW, Weldon PJ. 1993. β-Springene in the paracloacal gland secretions of the smooth-fronted caiman (*Paleosuchus trigonatus*). Biochem System Ecol 21:533–534.

Bender JC. 1943. Anatomy and histology of the female reproductive organs of *Habrobracon juglandis* (Ashmead) (Hym., Braconidae). Ann Entomol Soc Am 36:537–545.

Billen J, Morgan ED.1998. Pheromone communication in social insects: sources and secretions. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML, editors. Pheromone communication in social insects. Ants, wasps, bees and termites. Boulder, CO: Westview Press. p 3–33.

Billen JPJ, Jackson BD, Morgan ED. 1988. Secretion of the Dufour gland of the ant *Nothomyrmecia macrops* (Hymenoptera: Formicidae). Experientia 44:715–719.

Blomquist GJ, Tillman JA, Mpuru S, Seybold SJ. 1998. The cuticle and cuticular hydrocarbons of insects: structure, function, and biochemistry. In: Vander Meer RK, Blum MS. 1985. Alarm pheromones. In: Kerkut GA, Gilbert LI, editors. Comprehensive insect physiology, biochemistry and pharmacology, vol. 9. New York: Pergamon Press. p 193–224.

Breed MD, Espelie KE, Winston ML, editors. Pheromone communication in social insects. Ants, wasps, bees and termites. Boulder, CO: Westview Press. p 34–56.

Budzikiewicz H, Djerassi C, Williams DH. 1964. Structure elucidation of natural products by mass spectrometry. Steroids, terpenoids, sugars and miscellaneous classes, vol. 2. San Francisco: Holden-Day.

Burger BV, le Roux M, Spies HSC, Truter V, Bigalke RC. 1978. Mammalian pheromone studies III. (E,E)-7,11,15-trimethyl-3-methylenehexadeca-12,6,10,14-tetraene, a new diterpene analogue of beta-farnesene from the dorsal gland of the springbok, *Antidorcas marsupialis*. Tetrahedron Lett 5221–5224.

Burger BV, LeRoux M, Spies HSC, Truter V, Bigalke RC. 1981. Mammalian pheromone studies. 4. Terpenoid compounds and hydroxy esters from the dorsal glands of the springbok *Antidorcas marsupialis*. Z Naturforschung C 36:340–343.

Copland MJW, King PE.1971. The structure and possible function of the reproductive system in some Eulophidae and Tetracampidae. Entomologist 1971:4–28.

Cruz-Lopez L, Flavia E, Patricio LRA, Morgan ED. 2001. Secretion of stingless bees: the Dufour gland of *Nannotrigona testaceicornis*. J Chem Ecol 27:69–80.

Dani FR, Morgan ED, Turillazzi S. 1996. The Dufour secretion of *Polistes* wasp: chemical composition and possible involvement in nestmate recognition (Hymenoptera: Vespidae). J Insect Physiol 42:541–548.

Francis GW, Veland K. 1981. Alkylthiolation for the determination of double-bond positions in linear alkenes. J Chromatogr 219:379–384.

Gozansky TK, Soroker V, Hefetz A.1997. The biosynthesis of Dufour's gland constituents in queens of the honeybee (*Apis mellifera*). Invertbr Neurosci 3:239–243.

Guillot FS, Joiner RL, Vinson SB. 1974. Host discrimination: isolation of hydrocarbons from Dufour's gland of a braconid parasitoid. Ann Entomol Soc Am 67:720–721.

Haynes KF, Birch MC. 1985. The roles of other pheromones, allomones and kairomones in the behavioral responses of insects. In: Kerkut GA, Gilbert LI, editors. Comprehensive insect physiology, biochemistry and pharmacology, vol. 9. New York: Pergamon Press. p 225–255.

Hoffmeister TS. 2000. Marking decisions and host discrimination in a parasitoid attacking concealed hosts. Can J Zool 78:1494–1499.

Howard RW. 1993. Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson DW, Nelson DR, editors. Insect lipids: chemistry, biochemistry and biology. Lincoln, NE: University of Nebraska Press. p 179–226.

Howard RW, McDaniel CA, Blomquist GJ. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar). J Chem Ecol 4:233–245.

Howard RW, Baker JE. 2003a. Cuticular hydrocarbons and wax esters of the ectoparasitoid *Habrobracon hebetor*: ontogenetic, reproductive and nutritional effects. Arch Insect Biochem Physiol 53:1–18.

Howard RW, Baker JE. 2003b. Morphology and chemistry of Dufour glands in four ectoparasitoids: *Cephalonomia tarsalis*, *C. waterstoni* (Hymenoptera: Bethylidae), *Anisopteromalus calandrae*, and *Pteromalus cerealellae* (Hymenopteraa: Pteromalidae). Comp Biochem Physiol B. 135: 153–167.

Ibrahim SA, Avery JW, Weldon PJ, Wheeler JW. 1998. Age-class differences in lipids from the paracloacal glands of the American alligator (*Alligator mississippiensis*). Z Naturforschung C 53:201–209.

Jackson LL, Blomquist GJ. 1976. Insect waxes. In: Kolattukudy PE, editor. Chemistry and biochemistry of natural waxes. Amsterdam: Elsevier. p 201–233.

Jervis M, Kidd N, editors. 1996. Insect natural enemies: practical approaches to their study and evaluation. London: Chapman and Hall.

Jurenka RA, Subchev M. 2000. Identification of cuticular hydrocarbons and the alkene precursor to the pheromone in hemolymph of the female gypsy moth, *Lymantria dispar*. Arch Insect Biochem Physiol 43:108–115.

Keegans SJ, Billen J, Morgan ED, Gökçen OA. 1993. Volatile glandular secretions of three species of new World army ants, *Eciton burchelli*, *Labidus coecus* and *Labidus praeditor*. J Chem Ecol 19:2705–2719.

Krokos FD, Konstantopoulou MA, Mazomenos BE. 2001. Alkadienes and alkenes, sex pheromone components of the almond seed wasp, *Eurytoma amygdali*. J Chem Ecol 27:2169–2181.

Marris GC, Hubbard SF, Scrimgeour C. 1996. The perception of genetic similarity by the solitary parthenogenetic parasitoid *Venturia canescens*, and its effects on the occurrence of superparasitism. Entomol Exp Appl 78:167–174.

McGaughey WH, Beeman RW. 1988. Resistance to *Bacillus thuringiensis* in colonies of Indianmeal moth and Almond moth (Lepidoptera: Pyralidae). J Econ Entomol 81:28–33.

Mudd A, Fisher RC, Smith MC. 1982. Volatile hydrocarbons in the Dufour's gland of the parasite *Nemeritis canescens* (Grav.) (Hymenoptera: Ichneumonidae). J Chem Ecol 8:1035–1042.

Nakanishi K. 1962. Infrared absorption spectroscopy. Practical. San Francisco: Holden-Day.

Nelson DR. 1978. Long-chain methyl-branched hydrocarbons: occurrence, biosynthesis and function. Adv Insect Physiol 13:1–33.

Oldham NJ, Billen J, Morgan ED.1994a. On the similarity of the Dufour gland secretion and the cuticular hydrocarbons of some bumblebees. Physiol Entomol 19:115–123.

Oldham NJ, Morgan ED, Gobin B, Schoeters E, Billen J. 1994b. Volatile secretions of an Old World army ant, *Aenictus rotundatus*, and chemotaxonomic implications of army ant Dufour gland chemistry. J Chem Ecol 20:3297–3305.

Pouchert CJ. 1989. The Aldrich Library of Infrared Spectra, Vol. 3. Vapor Phase. 1st ed. Milwaukee, WI: Aldrich Chemical Company.

Quicke DLJ.1997. Parasitic wasps. London: Chapman and Hall.

Robertson PL.1968. A morphological and functional study of the venom apparatus in representatives of some major groups of Hymenoptera. Aust J Zool 16:133–166.

Schal C, Sevala V, Cardé R. 1998a. Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. Naturwissenschaften 85:339–342.

Schal C, Sevala V, Young HP, Bachmann JAS. 1998b. Sites of synthesis and transport pathways of insect hydrocarbons: cuticle and ovary as target tissues. Am Zool 38:382–393.

Syvertsen TC, Jackson LL, Blomquist GJ, Vinson SB. 1995. Alkadienes mediating courtship in the parasitoid *Cardiochiles nigriceps* (Hymenoptera: Braconidae). J Chem Ecol 21:1971–1989.

Van der Horst DJ, Weers PMM, Van Marrewijk WJ A. 1993. Lipoproteins and lipid transport. In: Stanley-Samuelson DW, Nelson DR, editors. Insect lipids: chemistry, biochemistry and biology. Lincoln: University of Nebraska Press p 1–24.

Vinson SB, Guillot FS. 1972. Host marking: source of a substance that results in host discrimination in insect parasitoids. Entomophaga 17:241–245.

Waterhouse JS, Hudson M, Pickett JA, Weldon PJ. 2001. Volatile compounds in dorsal secretions of the white-lipped peccary, *Tayassu pecari*, from Bolivia. J Chem Ecol 27:2459–2469.